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(57) Abstract

Disclosed is a method of diagnosing hepatocellular injury in a human patient, the method comprising the steps of: obtaining a sample of serum from the patient; assaying the sample to determine the amount of FBPase in the sample; comparing the amount of FBPase in the sample with the amount of FBPase in a previous sample from the same patient and/or with the amount of FBPase which might be expected for a healthy individual; and making a diagnosis based on the comparison, and a kit for performing the method.

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DIAGNOSIS OF HEPATOCELLULAR INJURIES USING SERUM FRUCTOSE-1,6-BIPHOSPHATASE

Field of the Invention

This invention relates to a method of diagnosing disease and specifically relates to a method of diagnosing liver damage in a human subject, and relates to a test kit for perfoming the method.

Background of the Invention

The conventional tests of "liver function", widely adopted, include measurements of serum levels of aspartate (AST) and alanine (ALT) transaminases, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), bilirubin, albumin and clotting factors. None of these tests is specific for liver disease, and elevated serum levels of the enzymes and bilirubin, as well as decreased concentrations of albumin and clotting factors, may occur in other conditions. For this reason, patterns of test results are often used to detect hepatocellular damage, loss of function or biliary obstruction. Even so, evaluation of liver disease can pose difficulties, owing mainly to the poor sensitivity of the routine liver function tests. This is exemplified by the delayed increase in serum transaminases following acute hepatocellular injury, as would occur, for example, following paracetamol poisoning.

Previous studies have shown glutathione S-transferase (GST) to be a very sensitive marker of acute hepatocellular injury. GST has been evaluated in the context of detection of acute allograft rejection in liver transplant recipients. Among these patients, the risk of rejection of the allografted organ is particularly high in the first few weeks after transplantation (Esquivel et al. 1985), and early diagnosis is essential in order to implement early measures that would limit the immune damage to the allograft (Trull et al. 1994; Rees et al. 1995; Hughes et al. 1997). GST is of particular value in the assessment of hepatocellular injury, due to its fast increase in serum following an acute episode. It also has a short in vivo half-life that results in a rapid fall in its serum concentrations in response to successful treatment (Trull et al. 1994), permitting withdrawal of potentially harmful immunosuppressants soon after resolution of the rejection episode. However, although of clinical benefit in diagnosis and management of patients, owing to the presence of different isoforms, as well as low enzymatic activity in serum, GST assays are based on immunological methods, which are still relatively time-consuming, labour-intensive and expensive. For these reasons, GST assay has not yet found a niche in routine clinical biochemistry.

In search of an enzymatic method that may offer the high sensitivity and specificity of GST in detecting acute liver damage, the inventors analysed a number of cytosolic and mitochondrial liver enzymes, including glucokinase, glutamine synthetase, carbamoyl phosphate synthase and pyruvate kinase, in preliminary studies using serum samples from patients who experienced acute hepatocellular damage. None of these tests proved sufficiently sensitive and sufficiently free of gross interference from serum to render it suitable for diagnostic use.

Another enzyme investigated by the inventors was fructose-1,6-bisphosphatase (EC 3.1.3.11; FBPase). FBPase is a gluconeogenic enzyme which, whilst predominately found in the liver, also occurs to a lesser extent in renal cortex, muscle and pancreas (Mizunuma & Tashima 1990; Saez et al. 1996). In muscle, the concentration of the enzyme, judged by its activity, has been reported to be very low (Mizunuma & Tashima, 1986). Both the concentration and the activity of the enzyme in liver are influenced by hormones that affect glucose homeostasis. Thus, insulin decreases FBPase gene expression in the rat (el Maghrabi et al. 1991) and increases the enzyme K_m in cultured hepatocytes (Ekdahl & Ekman 1987). Glucagon increases the V_{max} of the enzyme (Casteleijn et al. 1986), and both that hormone and adrenaline are capable of eradicating the effects of insulin on the enzyme K_m (Ekdahl & Ekman 1987).

Subcellular fractionation studies have revealed a large proportion of hepatocellular FBPase to be associated with subcellular particulate structures in the cytoplasm, suggesting that the enzyme may not be regarded as soluble in the classical sense (Saez et al. 1996).

Unlike GST, which has a broad distribution in the liver with a slightly greater perivenous prevalence, FBPase is predominantly localised in the periportal zones of the liver (Kress & Katz 1993; Saez et al. 1996). The pattern of enzyme release from perivenous and periportal zones may differ in various types of liver injury. For instance, whereas hypoxia or intoxication with halogenated hydrocarbons result in perivenous hepatic injury, during chronic aggressive hepatitis or allylalcohol poisoning, typical piecemeal necrosis is predominantly localised in the periportal zone (Thurman et al. 1986). Acute allograft rejection also often appears to begin in the periportal region, where FBPase is more prevalent.

Knowledge of changes in serum FBPase in liver disease is very limited. In their pioneering works, Kaldor & Schiavone (1968) and Nath & Gosh (1967), used crude methods for measuring serum enzyme activity. Several years later, von Rechenberg et al. (1984b) proposed the employment of serum FBPase measurements together with measurements of AST and glutamate dehydrogenase in an attempt to derive a diagnostic index in order to discriminate chronic persistent hepatitis from chronic aggressive hepatitis. Von Rechenberg et al stressed that the index was "not proposed for discriminating between healthy people and patients with inflammatory liver disease: this task is fulfilled by AST and ALT". An increase in the serum FBPase activities following administration of carbon tetrachloride to the rat was documented by Morata et al. (1990), whereas Jimenez-Jativa et al. (1992) reported that bile duct ligation decreased the serum enzyme levels.

Summary of the Invention

In a first aspect the invention provides a method of diagnosing hepatocellular injury in a human patient, the method comprising the steps of: obtaining a sample of serum from the patient; assaying the sample to determine the amount of FBPase in the sample; comparing the amount of FBPase in the sample with the amount of FBPase in a previous sample from the same patient, and/or with the amount of

FBPase which might be expected for a healthy individual; and making a diagnosis based on the comparison.

The method of the invention may be used to diagnose either chronic or acute hepatocellular injury. Conditions leading to chronic hepatocellular injury which might be suitable for diagnosis by the method of the present invention include, for example, cirrhosis, chronic active hepatitis, obstructive jaundice, and the like. Conditions leading to acute hepatocellular injury include, for example, acute viral hepatitis, ingestion or administration of toxic substances (especially toxic overdoses of pharmaceutical substances, such as paracetamol), and episodes of immune rejection of transplanted livers.

Those skilled in the art will appreciate that conventional tests of liver function, and other diagnostic methods (e.g. physical examination) may be used by a clinician in combination with the method of the invention in reaching a diagnosis. In addition, those skilled in the art will appreciate that a sample of plasma from the patient, rather than a serum sample, may be employed in the method of the invention with equivalent effect, although serum is much more commonly used in practice. Accordingly, the term "serum" as used herein is intended to refer equally to plasma.

Various methods of determining the amount of FBPase in a sample may be employed. For example, an immunological test (such as an ELISA) may be employed to determine the amount of FBPase antigen. More preferably however, a biochemical method is used (i.e. a method which relies on the biochemical properties of FBPase, rather than the immunological properties thereof), biochemical tests being generally quicker and cheaper to perform than immunoassays. Such biochemical assay methods allow for a determination of the amount of FBPase by measuring the amount of FBPase enzyme activity in the sample.

The assay method of the invention is preferably an enzyme linked biochemical assay method (i.e. an assay in which the enzymatic properties of FBPase are utilised as one of a series of linked reaction steps). Preferably the assay method is such that the assay result may be read spectrophotometrically (e.g. the reactions in the assay may produce a coloured product, and/or use up a coloured substrate, giving a colour change in the reaction mixture). The assay method may be for example, an end-point assay, in which the assay result is read at a particular time point (usually when the assay reaction has reached equilibrium). More preferably however, the assay method will comprise a kinetic assay, in which readings are taken at several time points. Such kinetic assays allow for determination of the rate of reactions, and are more widely used in clinical practice than end-point assays, being more sensitive and more informative. A suitable enzyme-linked biochemical test for FBPase activity has been disclosed in the prior art (von Rechenberg et al, 1984) and is further described in the examples below. Other biochemical assay methods for determining FBPase activity will be apparent to those skilled in the art.

The inventors have found that the method of the invention offers several advantages over the conventional biochemical assay (based on serum level of alanine transaminase [ALT], or aspartate transaminase [AST] activity) for liver function. In particular, the inventors have found that serum FBPase activity increases much more rapidly than serum ALT activity following hepatocellular injury, allowing earlier diagnosis. In addition, serum FBPase levels return to normal more rapidly, following successful treatment, than do serum ALT levels, so the progress of therapeutic regimes can be more closely followed. Furthermore, the inventors have found that serum FBPase level is a more sensitive indicator of acute hepatocellular injury than serum ALT, such that increased FBPase levels can be detected in some patients with liver injury whose serum ALT levels remain normal. The method of the invention is particularly applicable to the diagnosis and monitoring of acute hepatocellular injury, such as immune rejection episodes in liver transplant patients, partly due to the short in vivo serum half-life of FBPase.

The presence or absence of other diagnostic features will assist in diagnosis, but generally obtaining two or more serum samples from a patient, each of which shows an increased level of FBPase compared to a preceding sample, will be taken as indicative of hepatocellular injury. Alternatively, for example if no earlier serum samples from the patient are available, the result of the FBPase assay may be compared with a reference giving the amount of serum FBPase which might be expected for a healthy patient (e.g. matched for age, gender, race etc) - a value above the reference will be generally taken as indicating hepatocellular injury. The inventors consider that a typical maximum serum level of FBPase activity in a healthy human individual, under the test conditions described below (e.g. pH 7.5 etc.), is typically about 0.6 IU/L (conveniently in the range 0.58-0.65 IU/L) although those skilled in the art will recognise that this figure serves only as a guide, and clinical practitioners are free to determine what level they would consider as a typical maximum serum level of FBPase activity in a healthy human individual. In particular, conducting the assay under different test conditions will give different results, so a different reference range would be used. With the benefit of the present disclosure, those skilled in the art would be able to ascertain appropriate reference values for different assay conditions, by routine investigation.

In a second aspect the invention provides a diagnostic test kit for diagnosing hepatocellular injury in a human patient, the kit comprising reagents for measuring the amount of FBPase in a serum sample from the patient, the reagents being packaged in a container preferably together with instructions for use. The container may conveniently comprise cardboard packaging of a conventional nature.

The assay reagents and/or other components of the kit may be provided in liquid form (e.g. in solution) at ambient temperature or below (e.g. frozen). More conveniently the assay reagents and/or other test kit components will be provided as freeze-dried solids, for resuspension prior to use. In such an embodiment, the freeze-dried solids may be resuspended in distilled water or in appropriate buffers (which may be provided as part of the kit if desired).

The instructions for use provided with the kit will typically describe a method in accordance with the first aspect of the invention. The kit may also comprise one or more of the following components: positive and/or negative controls; reference samples; buffers; and quality assurance materials. Typical quality assurance materials may comprise serum (or plasma) samples containing a known amount of FBPase.

In some embodiments the kit will also comprise specific inhibitors of FBPase, so as to allow determination of amounts of non-specific phosphatase activity in the sample from the patient. The amount of non-specific phosphatase activity in the sample may then be deducted from the total enzyme activity so as to provide a result for specific FBPase activity. One such specific FBPase inhibitor suitable for inclusion in the kit is adenosine monophosphate (AMP).

An alternative approach is to employ substances which inhibit non-specific phosphatase activity in the sample due to serum alkaline phosphatase (e.g. bromoteramisole, BTO, which is also suitable for inclusion in the kit). The method of the invention may also comprise the use of FBPase inhibitors such as AMP, or alkaline phosphatase inhibitors such as BTO. Conveniently, samples may be tested in parallel, in the presence and in the absence of a relevant inhibitor, so as to provide a figure for FBPase-specific activity in the sample.

In one embodiment the assay reagents in the kit will normally comprise fructose-1, 6-bisphosphate at a known concentration. Conveniently the kit will further comprise additional reagents, for performing a "linked" assay, e.g. of the type known from the prior art. Accordingly the kit may also comprise as reagents: an isomerase (e.g. phosphoglucose isomerase); glucose-6-phosphate dehydrogenase (G-6-PDH), and NADP. In such a linked assay, FBPase activity is measured indirectly, being proportional to the rate of generation of NADPH, which may conveniently be determined spectophotometrically by measuring absorbance at 340nm. However, a number of other methods of assaying for FBPase activty are known, and so equivalent kits comprising other relevant reagents will be apparent to those skilled in the art. For example, an alternative embodiment may comprise the use of the enzymes phosphofructokinase (PFK), pyruvate kinase (PK), and lactate dehydrogenase (LDH), according to the linked rection scheme below:

(i) Fructose-1,6-bisphosphate $\xrightarrow{\text{FBPase}}$ Fructose-6-phosphate

(ii) Fructose-6-phosphate + ATP $\xrightarrow{\text{PFK}}$ Fructose-1,6-bisphosphate + ADP

(iii) Phosphoenolpyruvate (PEP) + ADP $\xrightarrow{\text{PK}}$ Pyruvate + ATP

(iv) Pyruvate + NADH $\xrightarrow{\text{LDH}}$ Lactate + NAD

(the concentration of NADH being measured spectrophotometrically at 340nm, and being directly proportional to the activity of FBPase when the concentrations of F-1,6-bisP, ATP, PFK, PEP, PK, NADH, and LDH are optimal)

Preliminary work conducted by the inventors has indicated that removal of zinc from serum by the use of specific or non-specific zinc chelating agents, such as 1,10-phenanthroline or EDTA, increases FBPase serum activity. The diagnostic kit in accordance with the invention may therefore comprise a zinc chelating agent, either incorporated as part of the medium containing assay reagents of the kit, or as a separate component for addition to test samples.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings in which:

Figure 1 is a graph (Absorbance at 340nm against time, in seconds), showing the kinetics of changes in absorbance of FBPase assay mixture. Absorbance values are shown over a 20-minute period after the assay reagents were mixed with 20 µl of each of 4 serum samples with various FBPase activities in a total volume of 150 µl. The reaction was carried out at 37°C in the absence of AMP.

Figure 2 is a graph of enzyme activity (IU/L) against length of time (in days) of storage of serum sample at 4°C, assayed in one analytical batch with fresh assay reagents.

Figure 3 is a graph of FBPase activity (IU/L) against time (in days), showing that assay result is not affected by storage of the assay reagents for up to 8 days at 4°C prior to performance of the assay.

Figure 4 is a graph of ratio (%) of observed/expected FBPase activity against % sample haemolysis (together with the corresponding concentration of haemoglobin (in gms/L)), and demonstrates that the presence of haemolysis causes an overestimation of FBPase levels.

Figure 5 is a scatter graph of serum FBPase level (IU/L) for different patient groups, the upper limit of normal FBPase level is denoted by the broken horizontal line.

Figure 6 is a chart showing the day when the relevant serum enzyme level showed a significant increase in liver transplant patients, relative to the day of treatment (day 0) for an episode of acute transplant rejection. The horizontal bar gives the 95% confidence level. The results shown in panel A (lower panel) are for those samples in which GST was measured, whilst the results in panel B were derived from all patients studied.

Figure 7 is a graph of serum level (expressed as a multiple of the upper limit of normal range, "ULN") against time (days) for various enzymes in a representative patient undergoing an episode of acute allograft rejection.

Figure 8 is a graph of serum level (expressed as a multiple of the upper limit of normal range, "ULN") against time (days) for various enzymes in a patient admitted to hospital following paracetamol overdose.

Examples

Example 1: Characterisation of Serum FBPase Assay

1.1 Serum FBPase measurement

All chemicals and reagents for serum FBPase enzyme assay and its evaluation were purchased from the Sigma Chemical Company (Poole, Dorset, England). Serum FBPase was measured enzymatically according to the method of von Rechenberg et al. (1984a). This employed a linked kinetic enzyme assay technique, according to the following reactions:

(i) Fructose-1,6-bisphosphate $\xrightarrow{\text{FBPase}}$ Fructose-6-phosphate

(ii) Fructose-6-phosphate $\xrightarrow{\text{isomerase*}}$ Glucose-6-phosphate

(iii) Glucose-6-phosphate $\xrightarrow{\text{G-6-PDH**}}$ Gluconolactone-6-phosphate

NADP → NADPH

* phosphoglucose isomerase

** Glucose-6-phosphate dehydrogenase

In the presence of optimal concentrations of the substrate, isomerase, G-6-PDH and the enzyme co-factors, the FBPase activity is directly proportional to the rate of generation of NADPH, which was measured by an increase in the absorbance at 340 nm. The serum enzyme activity was measured at 37°C by the use of a Monarch autoanalyser (Instrumentation Laboratories), programmed to measure the rate of change in the absorbance of the reaction medium over a 10-min period. The final reaction mixture contained 0.1 mol/L Tris, pH 7.5 (at 37°C), 0.625 mmol/L fructose-1,6-bisphosphate, 10 mmol/L $MgCl_2$, 5,000 IU/L phosphoglucose isomerase, 0.4 mmol/L NADP-Na, 500 IU/L glucose-6-phosphate dehydrogenase, 5.5 mmol/L 2-mercaptoethanol and 200 µl/L of 30% Brij 35.

The measurements were carried out both in the presence and in the absence of adenosine-5-monophosphate (AMP), which is a specific allosteric inhibitor of FBPase, at a final concentration of 1.25 mmol/L (adjusted to pH 7.5) in the reaction medium. The use of AMP allowed estimation of non-specific phosphatase activity, which was then subtracted from the total activity in order to give the FBPase activity of samples. The Monarch instrument was programmed to mix 75 µL of reagents (excluding the substrate and AMP), 20 µL of substrate (with or without AMP) and 20 µL serum in a total volume of 150 µL.

1.2 Kinetics of FBPase assay

By the use of the FBPase assay protocol described above, serum samples from a number of patients with liver disease were used to assess changes in absorbance at 340 nm at 60-second intervals over a 20-minute period after the start of each reaction. AMP was excluded from the assay reagents in order to assess the change in absorbance due to FBPase activity.

Figure 1 shows changes in absorbance over time for 4 serum samples over a 20-minute period after the samples and assay reagents (excluding AMP) were mixed. These and similar findings obtained from several other samples with various FBPase activities showed that there was a lag phase in the kinetics of the enzyme measurements. The rate of change of absorbance with time appeared to become more linear towards the end of the first 10-minute period. Regression analyses of the data presented in Figure 1 showed that the standard errors for the rate of change of absorbance after the first 10 minutes were 0.15% for the sample with the highest activity and 0.06% for each of the other 3 samples.

Based on these data, there were further but very small increases in the rate of change of absorbance with time after the first 10 minute period. Accordingly, when the rates of change of absorbance over a 5 minute period were used to calculate serum FBPase activities, a significant difference was found between the activities measured between times 10 and 15 minutes (15.41 ± 5.68; mean ± S.E.M.) and those between times 15 and 20 minutes (16.29 ± 5.84; mean ± S.E.M.) after the start of the reaction (n=15; P =0.0026).

This suggests that taking absorbance readings at a time window later than 10 to 20 minutes as employed here would give a more accurate measure of the FBPase enzyme activity. However, given that improvements in the accuracy of the measured enzyme activities obtained over a later time window would be very small, and that such time windows would decrease the working range of the assay due to substrate depletion in samples with high FBPase activity, it was deemed that taking absorbance readings 10 to 20 minutes after the start of the reaction would be an acceptable compromise for measurements of FBPase activity in serum samples. By the use of this time window under the conditions employed, the linearity of the rate of absorbance change was maintained with serum FBPase activities of up to 100 IU/L, but the window selected, within sensible limits, is not critical.

1.3 Effects of pH

The effects of hydrogen ion concentration on the measured FBPase activity were studied by the use of reagents that were adjusted to various pH values ranging from 7.2 to 7.8. Four serum samples from 4 patients were used for the evaluations. Similar studies were also carried out in the presence of bromotetramisole (BTO), which is an inhibitor of ALP. BTO was used at a final concentration of 125 µmol/L in the assay medium.

In summary, the inventors found that increasing the pH over the range 7.2 to 7.8 units increased the measured activity of the FBPase assay. As the pH was increased, there were increases both in the measured total activity and blank activity (in the presence of AMP).

Most of the non-specific phosphatase activity in the FBPase assay is thought to be due to ALP in serum (von Rechenberg et al. 1984a). Therefore, it is possible that at least part of the increase in the

measured activity observed with an increase in the pH may to be due to interference from ALP, which has a high optimum pH. For that reason Bromotetramisole (BTO; oxalate salt), which is a specific inhibitor of ALP, was used at a final concentration of 125 µmol/L to reassess the effects of pH on FBPase activity. The results showed ALP inhibition to have a small but incomplete limiting effect on the increase in measured FBPase activity with an increase in the pH.

As a means of limiting the measured non-specific activity a standardised pH value of 7.5 was chosen, in agreement with that used in previously published work (Jimenez-Jativa et al. 1992), to measure serum FBPase activity in subsequent experiments. However, as with the time window, the pH conditions employed are not critical as long as they are within sensible limits. Those skilled in the art will recognise however that adoption of different pH conditions for the assay will produce different absolute values for FBPase than those detailed in the present specification.

1.4 Linearity at different serum concentrations

The recovery of the enzyme activity was measured by the use of 3 serum samples with FBPase activities close to 100 IU/L, and re-measurement of the activities at various dilutions of the samples. The serum samples were from patients with acute liver disease and were diluted serially in either phosphate-buffered (pH 7.4) normal saline solution (PBS) or in serum from patients with no biochemical evidence of liver disease.

Dilution of samples in PBS produced results which matched those from dilutions in serum. Generally, samples with activities of greater than 100 IU/L were diluted in order to obtain a more accurate estimate of the magnitude of increase in enzyme activity (v.i. for reference range of FBPase in serum) and also to enable assessment of the direction of change in the activities over time in subsequent samples. The results showed the recoveries to average 112.3, 100.6 and 99.1 % of the expected values when the samples were diluted 1 in 4, 16 and 64 in serum respectively. The corresponding values when the samples were diluted similarly in PBS were 102.6, 83.7 and 97.4 % of the expected values respectively. Diluting the samples in PBS gave generally lower results than those obtained when the samples were diluted in serum. Nevertheless, these findings suggest that the assay performs well when dilution of samples with very high activities is required.

1.5 Stability of serum FBPase *in vitro*

Aliquots of 6 fresh serum samples were stored at -20°C. Over a period of one week, separate aliquots of the samples were thawed out on different days and stored at 4°C. At the end of this period, the FBPase activities of the all aliquots that had been stored at 4°C for a period of up to 9 days were measured in one analytical batch using freshly-prepared reagents.

The results are shown in Figure 2. There was no significant change in the enzyme activity of the samples over the first day of storage. Over a period of one week, however, there was a decrease in the activities, which averaged 26% of the activity measured at the outset. The FBPase activities (mean ±

S.E.M.), as a percentage of those on day 0 for the 6 samples, were 102 ± 3.6% on day 1, 82.0 ± 3.4% IU/L on day 5 and 60.3 ± 6.5% on day 9. These findings indicated a progressive deterioration of serum FBPase with time after a day of storage of specimens at 4°C.

The effects of temperature stress on the stability of FBPase in serum were investigated by subjecting different aliquots of a serum sample with a high FBPase activity to increasing numbers (up to 5) of freezing and thawing cycles, and measuring the enzyme activities of the aliquots in one analytical batch. The results are shown in Table 1 below. The table demonstrates that there was no loss of FBPase activity following freeze/thaw of samples.

Table 1

Freeze-thaw cycles	FBPase activity (IU/L)	Observed/expected activity (%)
0	93.3	100.0
1	91.8	98.4
2	97.4	104.4
3	94.0	100.8
4	92.3	98.8
5	91.3	97.9

In order to assess the stability of serum FBPase in frozen samples over time, 4 serum specimens with various enzyme activities were aliquoted and stored at -20°C. Over a period of up to 4 months, individual aliquots of the samples were thawed out and assayed at few-day intervals with the same batch of assay reagents that had been kept frozen since preparation.

The inventors found that there was no significant change in the measured activity of the samples over the first 2 months of the storage. In one sample that was stored for a period of 120 days, however, a gradual decrease in the measured activity was noted after the first 2 months, such that the values obtained between days 82 and 120 were significantly lower than those from days 1 to 67 (mean ± S.E.M. values of 1.76 ± 0.04 and 2.24 ± 0.10 respectively; P = 0.0003)

1.6 Stability of assay reagents at 4°C

Aliquots of 3 serum samples that were not more than one-day old were stored at -20°C. On separate days over a period of one week, one aliquot of each serum sample was thawed and its FBPase activity measured with reagents that had been stored at 4°C. The results are shown in Figure 3. In summary, no change was detected when the assay reagents were stored at 4°C for up to 8 days.

1.7 Analytical interference

A number of experiments were performed to investigate the sensitivity of the FBPase assay to possible interference by substances which might be expected to be regularly or occasionally present in clinical serum samples.

Bilirubin

Since patients with hepatocellular damage often have icteric serum, the effect of bilirubin on serum FBPase measurement was studied. Serum samples with various FBPase activities were spiked with either PBS (controls) or serum that contained bilirubin at concentrations of up to 876 µmol/L (and FBPase activities of less than 0.76 IU/L) before FBPase measurements were undertaken.

In summary, the presence of high bilirubin concentrations had no effect on the FBPase level determined by the assay. This was confirmed by dilution of serum samples which had high bilirubin content, the dilution series giving consistent values for FBPase.

Haemolysis

The interference of haemolysis in many biochemical assays in routine use is a well-known phenomenon (Brady & O'Leary 1998; Donnelly 1998), and was accordingly investigated to see if there was any effect on the FBPase assay.

Blood samples that had been collected in EDTA-containing tubes (for separate routine laboratory work taken from diabetic patients with no biochemical evidence of liver disease) were used for the preparation of a red cell haemolysate. The blood samples were centrifuged for 10 minutes at 3,000 g, the supernatants were decanted, and the cells suspended in PBS. The suspensions were then recentrifuged and the washing step was repeated 5 times. After a final centrifugation, the cells were lysed in distilled water, which was added to adjust the volume to twice that of the original samples. The red cell lysates were then centrifuged at 17,000 g for 10 minutes, and the supernatants used for the assessment of interference of haemolysis in the FBPase assay.

The red cell lysates were diluted in PBS to give solutions with haemoglobin concentrations ranging from 2.0 to 80 g/L. These corresponded to a haemolysis of approximately 1.0 to over 50% of the red cells. The dilutions of red cell lysates were added to serum samples with various enzyme activities in a ratio of 1:3. The FBPase activities were measured in these specimens and compared with those in the corresponding serum samples which were diluted in a similar fashion with PBS. The results are shown in Figure 4.

Despite the absence of significant amounts of measurable FBPase activity in red cell lysate (activities of less than 1 IU/L), haemolysis increased the measured serum FBPase activity, indicating an interference from the red cell constituents. The data presented in Figure 4 demonstrate that at a degree of haemolysis that corresponded to lysis of 1% of the red cells in the sample, the increase in the serum

FBPase activity was about 40%. It should be emphasised that the amount of haemolysis in clinical samples is typically less than 0.5%.

In order to assess the nature of the interferent(s), red cell lysates were dialysed 3 times, each for 4 hours at 4°C, against a 100-fold volume of PBS. The effect of the dialysed haemolysates on the enzyme assay was then investigated. In a separate experiment, red cell lysates were placed in a 56°C water bath for 10 minutes, and the influence of the heat-treated lysates on serum FBPase activity was assessed. The results of these experiments indicated that the interferent(s) was retained by dialysis membrane, and that it was heat-denaturable. These suggested that the interferent(s) was most probably a protein and likely to be an enzyme.

Since red cells contain high concentrations of G6PDH and moderate levels of phosphoglucose isomerase, both of which are used in the FBPase enzyme assay, the effects of excess amounts of these two enzyme on the reaction rates used to measure FBPase activity was evaluated. However the inventors found that neither the concentration of G6PDH nor isomerase used in the assay was limiting, suggesting the sources of the red cell interference to be other than these two enzymes.

The influence of adenylate kinase (AK; myokinase; EC 2.7.4.3), which transfers phosphate moieties between adenine nucleotides, was also investigated. Exogenous AK added to serum increased the measured rate of FBPase activity (data not shown). However, the increase in the measured rate was not saturable in the AK concentration range of up to 10 kIU/L studied. When P^1P^5-di(adenosine-5') pentaphosphate (Ap5A), a competitive inhibitor of AK, was used in the assay mixture at a final concentration of 1 µmol/L (reported to inhibit 98% of AK activity; Lienhard et al. 1973) and 10 µM, no reduction in the magnitude of the effect of AK on the measured activity was noted. However, Ap5A at the higher concentration decreased the effects of haemolysis on the measured FBPase activity, but did not manage to eradicate the interference completely (data not shown).

Since red blood cells are rich in the enzymes of the pentose phosphate pathway, the possibility of involvement of the enzymes of that pathway as an source of extra NADPH was investigated. This was done by inhibition of phosphogluconate dehydrogenase by the use of pyridoxal-5-phosphate at a final concentration of 0.43 mmol/L. The results indicated that the interference from the red cell lysate could not be inhibited by the use of the inhibitor.

Whilst it appears difficult to prevent haemolysis-mediated interference in the FBPase assay, in practice this should not present a serious difficulty as haemolysed serum samples are easily recognisable at serum haemoglobin concentrations of around 0.5gms/L (Sonntag, 1986), so institution and implementation of proper checks should safeguard against overestimation of FBPase activity caused by haemolysis.

Example 2

Reference range

In order to establish a working reference range for use in clinical evaluation of serum FBPase, samples from healthy subjects were studied.

Serum FBPase activity measurements in samples from 94 healthy blood-donors, of either gender, aged between 18 and 65 years, indicated a range in adults from 0.03 to 0.65 IU/L. The frequency plot of the values showed a normal distribution of the data with a small negative skewness (the median value was 0.28 IU/L). The detrended normal P-P plots (deviation from normal versus observed cumulative probability) suggested that transformation of the data offered no advantage in compensating for the skewness for establishing a reference range. From this data, a reference range of 0.03 - 0.58 IU/L was derived. Since only increases in the enzyme activity are of clinical significance, and taking into account the relative imprecision of the assay at very low serum enzyme activities, for practical purposes a reference range of less than 0.58 IU/L was adopted for evaluation of the results from patients in subsequent studies.

Example 3

Specificity of FBPase assay

The effects of chronic renal failure, as well as cardiac and skeletal muscle damage on serum FBPase activities were assessed. Serum enzyme activities were also measured in conditions where gluconeogenesis was affected. These included poorly-controlled diabetes mellitus, and untreated Addison's disease and Cushing's.

Chronic renal failure

The FBPase activity was measured in serum samples from 22 patients who had chronic renal failure, with serum creatinine concentrations ranging from 800 to 1262 µmol/L, but no biochemical evidence of liver disease. The results were compared with the reference range for the serum enzyme assay.

Acute myocardial infarction (AMI)

Serum samples from 23 patients with AMI were used for measurement of FBPase activity. The criteria for selection of these patients were a total serum CK (creatine kinase) activity of greater than 250 IU/L (reference ranges were 24-195 IU/L for males and 24-170 IU/L for females), a CKMB (MB isoform of CK) fraction of greater than 6% of the total CK and no biochemical evidence of liver disease. When more than one sample from a patient was available, the sample with the highest CK activity was chosen. The serum CK levels ranged from 250 to 3744 IU/L with a median value of 444 IU/L.

Myolysis

The effects of acute skeletal muscle cell damage on serum FBPase was studied in 20 patients, who presented with serum CK activities of greater than 350 IU/L, and a CKMB fraction that was at no time

more than 6% of the total CK. The CK values ranged from 350 to 312,000 IU/L with a median value of 560 IU/L.

Diabetes

Serum FBPase was measured in the blood samples from 21 diabetic patients who had a blood haemoglobin A_{1c} fraction that was greater than 10% of the total haemoglobin A_1. The plasma glucose concentrations in the blood of these patients ranged from 10.9 to 25.8 mmol/L at the time of sampling. These patients had no biochemical evidence of liver disease based on their conventional liver function test results.

Addison's disease

Serum samples from 20 patients with untreated Addison's disease were assayed for FBPase. The patients had a serum cortisol response to synacthen that was less than 450 nmol/L at 30 and 60 minutes after the dose, with an increment of less than 200 nmol/L compared with the baseline value. The baseline samples were used for FBPase measurements.

Cushing's

Serum samples from 13 patients with untreated Cushing's disease or Cushing's syndrome were assayed for FBPase activity. All the patients had 24-hour urinary free cortisol excretions of greater than 270 nmol and had no biochemical evidence of liver disease. The results of these studies are shown below in Table 2 and presented graphically in Figure 5.

Table 2

Condition	*n*	Serum FBPase (IU/L)			Number with values > ULN
		Range	Median	Mean ± S.E.M.	
CRF	22	0.04-0.66	0.39	0.39 ± 0.03	2
AMI	23	0.06-0.59	0.33	0.33 ± 0.03	1
Myolysis	20	0.01-0.60	0.30	0.32 ± 0.04	2
DM	21	0.01-0.84	0.38	0.38 ± 0.04	1
Addison's	20	0.01-0.64	0.32	0.33 ± 0.04	1
Cushing's	13	0.01-0.58	0.26	0.30 ± 0.05	0

Table 2. Serum FBPase activities in different disease states.

Abbreviations: *n* = the number of patients in each group; ULN = Upper limit of normal (0.58 IU/L, based on the established reference range); CRF = Chronic renal failure; AMI = Acute myocardial infarction; DM = Diabetes mellitus

The results showed that 112 out of the 119 patients studied had serum FBPase values that were within the reference range. Of the 7 patients who had serum FBPase activities greater than the upper reference limit of the assay, one patient with chronic renal failure had a systemic infection at the time of sampling, with a serum C-reactive protein concentration of 200 mg/L, and the Addisonian patient with elevated serum FBPase was found to be also diabetic. Among the other patients with elevated serum FBPase activity, no clinical or biochemical history that might have been relevant could be found. The mean values for serum FBPase were comparable in the various groups. However, the patients with chronic renal failure and diabetes had the highest median values compared with the other 4 groups (Table 3).

Example 4

Comparison of serum FBPase with ALT and GST for the detection of hepatocellular injury in liver transplant recipients

Routine sequential samples from 25 liver transplant recipients who had sustained an episode of rejection of the allografted organ were analysed for FBPase and other liver function tests. GST measurements were also carried out on the samples from 13 of the patients. Rejection episodes were diagnosed either by biopsy or, when biopsy was contraindicated, on clinical grounds based on deteriorating liver function tests, increased prothrombin time and a rise in eosinophil count (Hughes et al., 1998).

Of the 25 patients studied, 20 underwent a liver biopsy, and, of these, 15 received specific treatment for acute hepatocellular rejection, usually in the form of 3 daily doses of 1 g intravenous methylprednisolone. Five of the 25 patients received treatment for acute rejection but for clinical reasons were not biopsied in the period immediately before or after treatment. In case of 4 of the treated patients, the biopsies were carried out only after the completion of the course of treatment, when contraindications for liver biopsy, such as thrombocytopaenia, had diminished.

In order to define a significant increase in serum FBPase, ALT or GST in association with a rejection episode, a set of simple criteria were adopted. Thus, the beginning of a significant increase in serum activity of an enzyme was defined as the time when there was an increase of at least 50% in serum activity in relation to one of the previous 2 values. However, an increase of at least 25% was considered significant if there had previously been a continuous decrease in serum enzyme level (a decrease of at least 10% per day for at least 2 days). A peak was defined as the maximum value in serum activity of an enzyme reached after a significant increase, when there was a subsequent fall in the enzyme activity for at least 2 days.

Changes in serum FBPase, ALT and GST were compared in association with acute hepatocellular rejection and the time of start of specific treatment for the episodes. An association was considered to be present when there was an increase in serum enzymes over the period from 4 days prior to 3 days after a biopsy-proven rejection or specific treatment. The rate of the rise in enzyme levels, the time of the peak of the studied enzymes in relation to one another, and the rate of decrease of the enzymes in serum were also studied.

Of the 25 episodes of acute hepatocellular rejection studied, a significant increase in serum ALT activity was recorded in only 15. However, serum FBPase activity was found to be raised in relation to 24 rejection episodes. The patient whose serum FBPase did not increase also showed no increase in serum ALT. Serum GST was elevated in 12 of the 13 episodes where it was measured. Serum FBPase, but not ALT, rose in the patient who had no increase in serum GST.

According to these results, the sensitivity of serum ALT for the detection of acute hepatocellular rejection was 60%. At 96% for FBPase and 92% for GST, the sensitivities of tests based on these enzymes are considerably better than that based on serum ALT. Compared with ALT, serum FBPase began to increase on average 0.8 days (range -1 to +3 days; 95% confidence interval = 0.4-1.3 days) earlier in association with an acute rejection episode, and its peak values were reached on average 0.9 days sooner (range -1 to +2 days; 95% C.I. = 0.2 - 1.5 days). Comparison of serum FBPase and GST levels showed that serum GST began to increase earlier in 2 patients, by 1 and 3 days. In the case of 2 other patients, however, the increase in serum GST occurred 1 day later than that of serum FBPase. Among the other 8 patients that showed a significant increase in GST in serum, the beginning of an increase in serum FBPase coincided with that of GST. Peak serum FBPase and GST were also reached on the same day in all the patients except one, in whom serum GST peaked 2 days in advance of serum FBPase. There was no relationship between the heights of the serum peaks of the three measured enzymes.

The beginning of significant increases in serum levels of the three enzymes in relation to the start of specific treatment for acute rejection have been compared in Figure 6. Among patients in whose samples GST was measured, serum FBPase and GST began to increase on average 0.6 and 0.7 days earlier than serum ALT respectively. Two patients in this group did not show any significant increase in serum ALT in relation to a rejection episode. There was no increase in serum ALT activity, but a significant increase in serum FBPase activity, in seven rejection episodes. In the rest of the patients, serum FBPase increased on average 0.9 days earlier than serum ALT (95% C.I. = 0.2-1.6 days).

Figure 7 illustrates changes in serum levels of FBPase, GST and ALT in a single representative patient during an episode of acute allograft rejection. The horizontal bar indicates the duration of treatment for rejection with augmented immunosuppression. Levels of FBPase are denoted by the round symbols, GST by the lozenge symbols, and ALT by the square symbols.

The median and the average rates of increase and decrease for the three serum enzymes have been shown in Table 3. Compared with ALT, serum FBPase activity increased faster in serum in relation to a rejection episode, and decreased more rapidly following the start of treatment. Compared with GST, however, FBPase had a slower average rate of increase and a slower median rate of decline in serum.

Table 3 (below). Comparisons of the rates of increase and decrease in serum ALT, FBPase, and GST in relation to episodes of acute allograft rejection in liver transplant recipients. The rates of increase and decrease are average rates per day and relate to those from the start of the rise to the peak and from the peak to a nadir or to the end of the sampling period, whichever occurred sooner, for the corresponding serum enzymes.

Table 3

Enzyme Assayed	*n*	Median increase	Rate of increase	Median decrease	Rate of decrease
ALT	16	60.3%	132.9%	12.4%	12.5%
FBPase	24	88.0%	524.3%	19.4%	45.0%
GST	12	128.7%	1402.7%	25.7%	21.1%

Example 5

Comparison of Serum FBPase and ALT in Patients with Paracetamol Overdose

A total of 32 patients who had taken an overdose of paracetamol were studied. All of them had a serum paracetamol concentration of greater than 0.1 mmol/L in the first blood sample that was taken after admission. The period of hospital stay for these patients was between 1 and 13 days. Changes in serum activities of FBPase and ALT were compared, and the data were analysed for the presence of, as well as the rate of rise in, the serum enzyme concentrations, the time of the peak of the studied enzymes in relation to one another, and the rate of decrease of the enzyme activities in serum. The start of an increase in the serum enzymes was defined as the time when there was greater than a 50% increase in the activity in relation to the previous result. A peak was defined as the maximum value in serum activity of an enzyme reached after a significant increase, after which there was a fall in the enzyme activity.

Serum FBPase and ALT measurements showed that at least one of the two enzymes was increasing in serum from 14 out of the 23 patients from whom more than one sample was received. There was no rise in serum FBPase in one of these 14 patients, compared with 5 who did not show an increase in serum ALT. The rate of the rise of serum FBPase was on average 4642% per day (median 1167%), compared with an average rate of rise of 351% per day (median 188%) for serum ALT.

In one patient, the peak serum FBPase was reached one day earlier than that for ALT. In another patient, the peak in serum FBPase was reached 2 days before the end of the sampling period, whereas no peak in serum ALT was observed during the period when samples were taken. When serum FBPase was decreasing, the average rate of decline was 54.4% per day (median 54.5%), compared with an average rate of decrease of 27.0% per day (median 28.1%) for serum ALT.

In the 23 patients from whom more than one serum sample was received, serum FBPase did not increase above the upper limit of the reference range in only one patient, in whom both serum FBPase and ALT were decreasing when the first sample was received. However, serum ALT activity was normal in all serum samples from 3 patients from whom serial samples were received.

In the case of 9 out of the 32 patients studied, only one serum sample was received from each patient. In none of these samples was there an increase above the reference range for either serum FBPase or ALT.

Data from a single representative patient are shown in Figure 8. The figure shows changes in serum levels of FBPase (crossed symbols) and ALT (round symbols) associated with paracetamol overdose in a patient who was admitted to a hospital casualty unit on day 1.

Discussion

The data described here demonstrate that serum FBPase can be useful as a marker of hepatocellular injury, and may offer a sensitivity close to that of GST, and greater than that of conventional assays for ALT or AST, for the detection of hepatocellular injury. The enzyme assay is easily amenable to automation, which is a requirement for modern methods in routine and emergency clinical biochemistry. Serum FBPase is stable enough at 4°C for retrospective analyses to be carried out on samples that are not more than one day old. After that time, the activity of the enzyme in serum decreases progressively.

From these studies, serum FBPase also appears to have high specificity for liver disease.

Compared with ALT, serum activities of FBPase were found to increase on average about one day earlier in association with episodes of rejection of allografted liver. In all but two patients, the increase in serum FBPase levels occurred in phase with (and in two cases earlier than) those of serum GST. The differences observed in the patterns of increase in serum concentrations, together with the fact that there was no relationship between the height of the peaks of the three enzymes relative to their normalised reference ranges, may be due to the differences in the patterns of distribution of these enzymes within the liver, the site of hepatocellular injury and release ratios of the three enzymes.

There was also a much more rapid decline in serum FBPase compared with ALT. The decreases in serum FBPase appeared to occur in phase with those of serum GST, with similarities in the rates of

decrease of both enzymes. These suggest that the in vivo serum half-life of FBPase in serum is much closer to that of GST (Trull et al. 1994) than the much longer half-life for ALT. There are many potential advantages in the use of an enzyme with a short half-life for the detection of liver damage. Due to the fact that new steady states in serum enzyme levels are reached faster, the serum enzyme activities at any time reflect more accurately the presence or the absence of recent episodes of liver injury (Hughes et al. 1997). Importantly, since the serum levels normalise much faster after such an episode, any subsequent liver injury, and particularly a smaller one, would be more easily detected when measurement of an enzyme with a shorter half-life is employed.

In the study of patients admitted for paracetamol overdose, FBPase appeared to increase in a greater number of patients compared with serum ALT. This increased sensitivity may partly be due to the faster rate of increase of serum FBPase than that of ALT.

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Claims

1. A method of diagnosing hepatocellular injury in a human patient, the method comprising the steps of: obtaining a sample of serum from the patient; assaying the sample to determine the amount of FBPase in the sample; comparing the amount of FBPase in the sample with the amount of FBPase in a previous sample from the same patient and/or with the amount of FBPase which might be expected for a healthy individual; and making a diagnosis based on the comparison.

2. A method according to claim 1, wherein the amount of FBPase in the sample is assayed by biochemical measurement of FBPase activity.

3. A method according to any one of claims 1 or 2, wherein FBPase activity is assayed by an enzyme-linked biochemical assay.

4. A method according to any one of the preceding claims, wherein FBPase activity in the sample is assayed by an enzyme-linked biochemical assay and the result of the assay is determined spectrophotometrically.

5. A method according to any one of the preceding claims, wherein a diagnosis of hepatocellular injury is made if the amount of FBPase in the sample exceeds the upper limit of a reference range established for healthy subjects.

6. A method according to any one of claims 1-4, wherein a diagnosis of hepatocellular injury is made if the amount of FBPase in the sample exceeds the amount of FBPase in a previous sample from the same patient.

7. A method according to any one of the preceding claims, for diagnosing hepatocellular injury caused by an acute condition.

8. A method according to any one of the preceding claims, for diagnosing an episode of allograft rejection in a liver transplant recipient.

9. A method according to any one of the preceding claims, comprising the use of one or more of the following as an assay reagent: fructose-1,6-bisphosphate; an isomerase; glucose-6-phosphate dehydrogenase; and NADPH.

10. A method according to any one of the preceding claims, comprising the use of a specific FBPase inhibitor, and/or a serum alkaline phosphatase inhibitor.

11. A method according to any one of the preceding claims, comprising the use of adenosine monophosphate and/or bromoteramisole.

12. A method according to any one of the preceding claims, comprising the use of a specific or non-specific zinc chelating agent such as 1,10-phenanthroline or EDTA.

13. A diagnostic test kit for diagnosing hepatocellular injury in a human patient, the kit comprising reagents for measuring the amount of FBPase in a serum sample from the patient, the reagents being packaged together in a container.

14. A kit according to claim 13, further comprising instructions for use according to the method of any one of claims 1-12.

15. A kit according to claim 13 or 14, further comprising one or more of the following components: positive and/or negative controls; reference samples; phosphatase inhibitors; zinc chelating agents; buffers; and quality assurance materials.

16. A kit according to any one of claims 13, 14 or 15, wherein the reagents and/or other components of the kit are provided as freeze-dried solids.

17. A kit according to any one of claims 13-16 comprising fructose-1,6-bisphosphate at a known concentration.

18. A kit according to any one of claims 13-17, further comprising one or more of the following: an isomerase; glucose-6-phosphate dehydrogenase; and NADPH.

19. A kit according to any one of claims 13-18, comprising a specific FBPase inhibitor.

20. A kit according to any one of claims 13-19, comprising adenosine monophosphate.

21. A kit according to any one of claims 13-20, comprising a serum alkaline phosphatase inhibitor.

22. A kit according to any one of claims 13-22, comprising bromoteramisole.

23. A method substantially as hereinbefore described.

24. A diagnostic kit substantially as hereinbefore described.

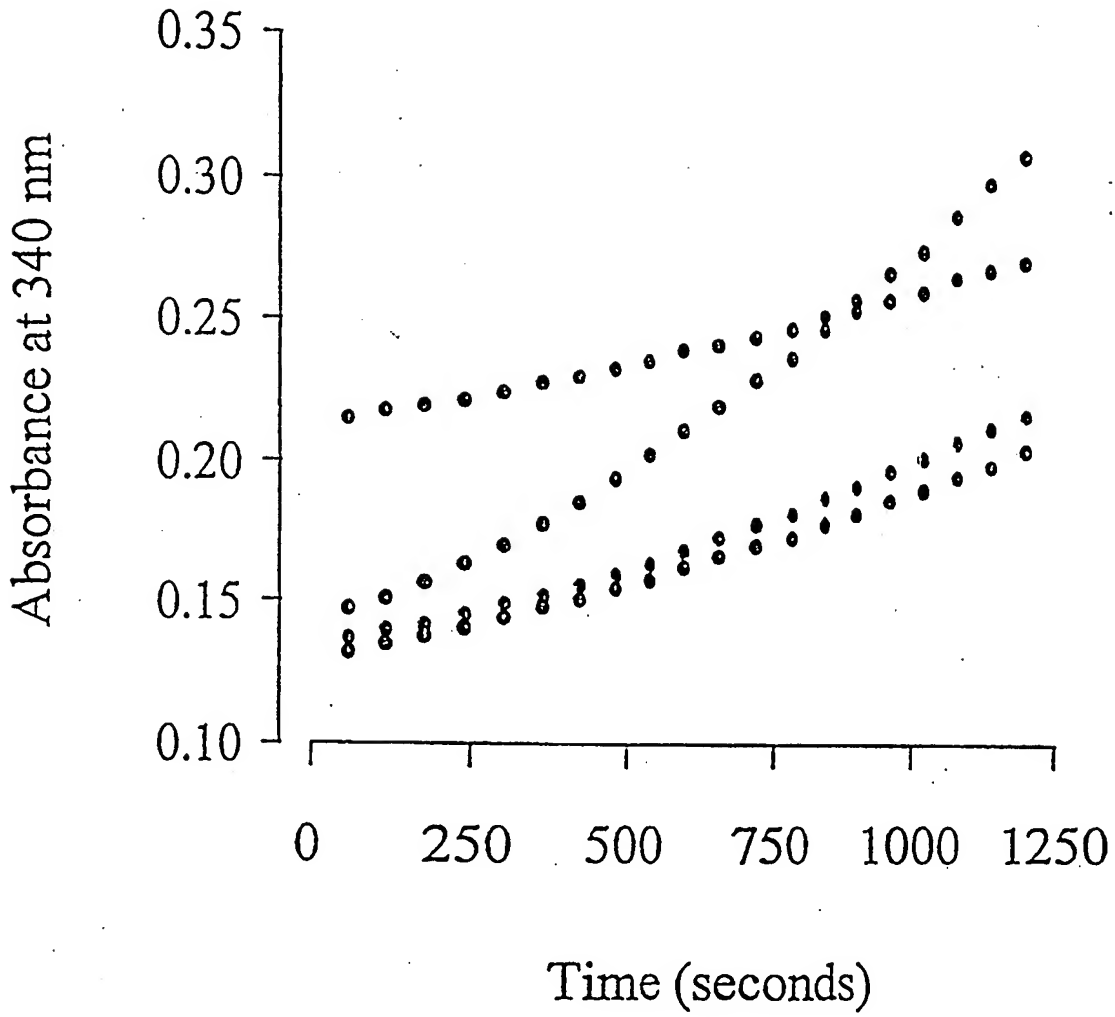

0.35
0.30
0.25
0.20
0.15
0.10
Absorbance at 340 nm
0
250
500
750
1000
1250
Time (seconds)

Fig. 1

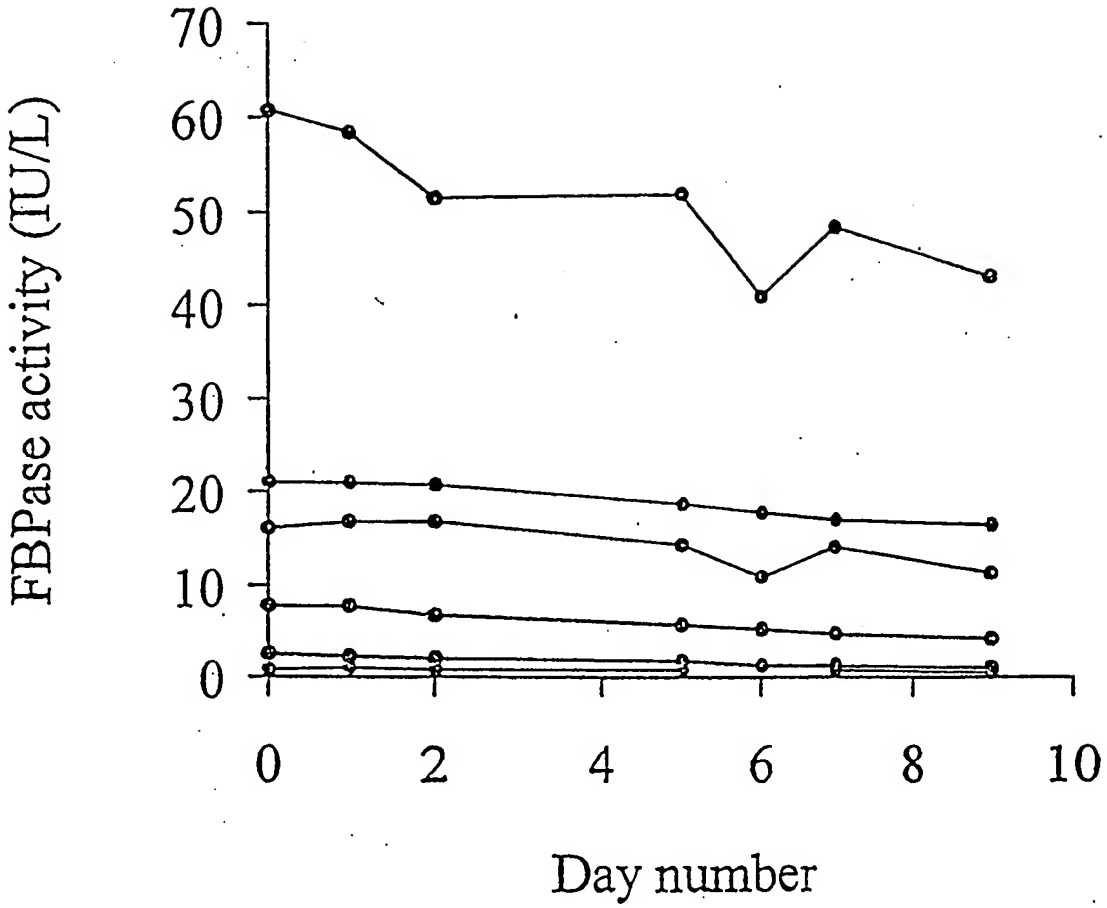


Fig. 2

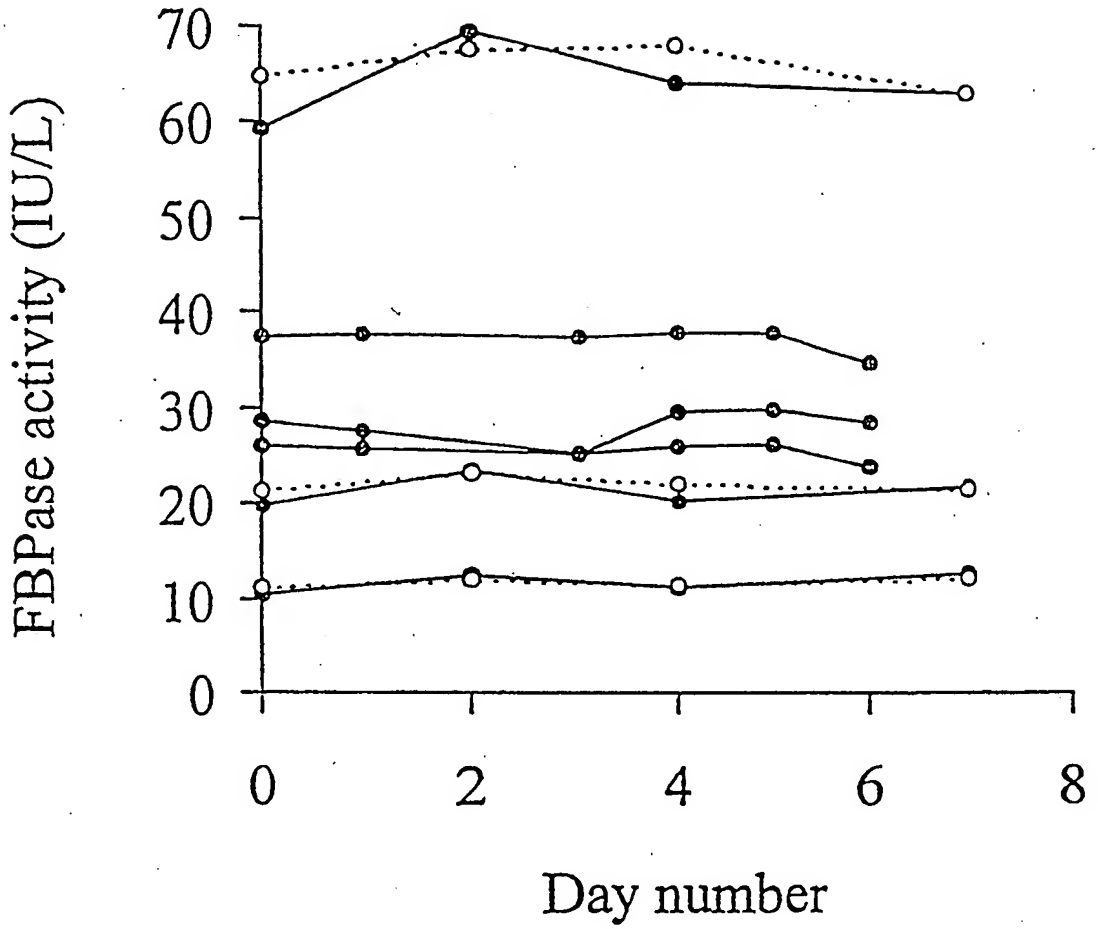
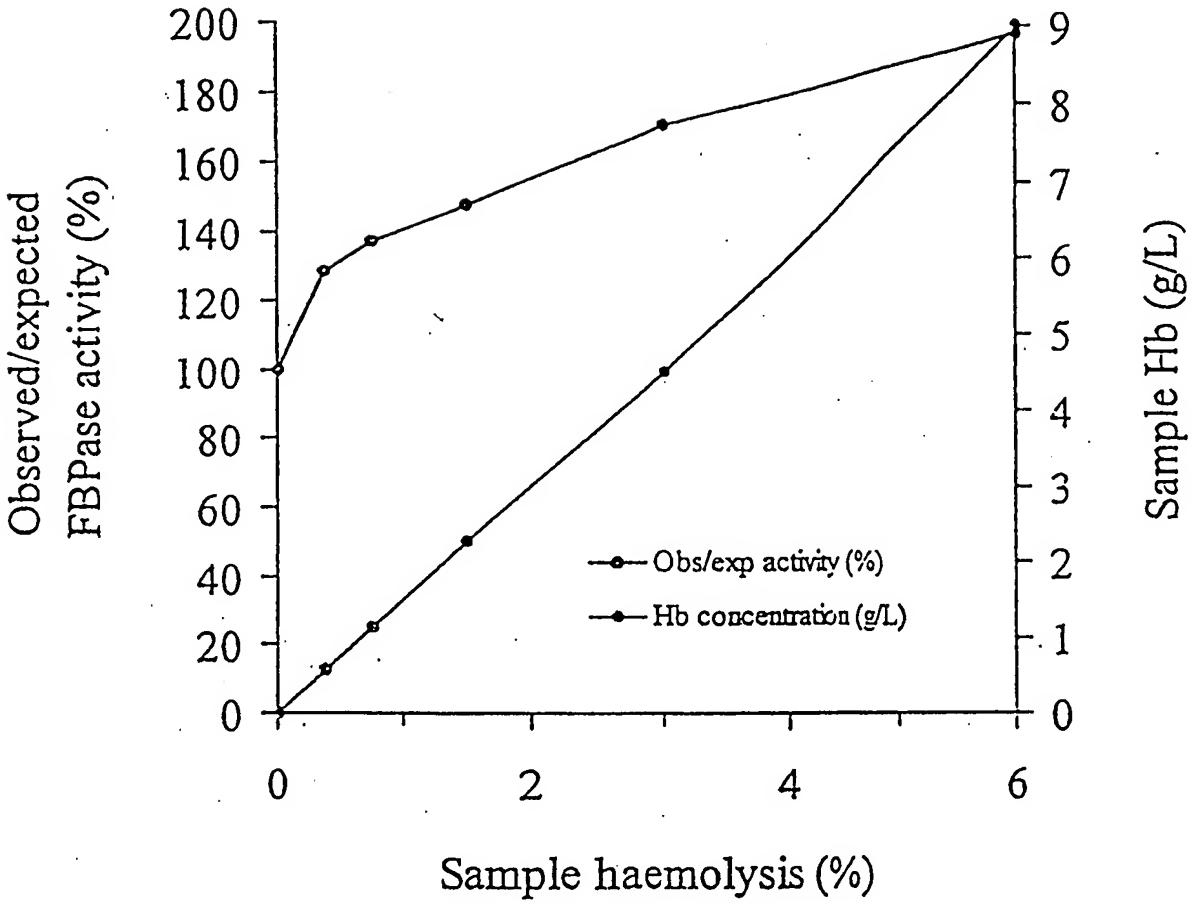


Fig. 3


Observed/expected FBPase activity (%)
Sample Hb (g/L)
Sample haemolysis (%)
Obs/exp activity (%)
Hb concentration (g/L)
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180
160
140
120
100
80
60
40
20
0
9
8
7
6
5
4
3
2
1
0
0
2
4
6

Fig. 4

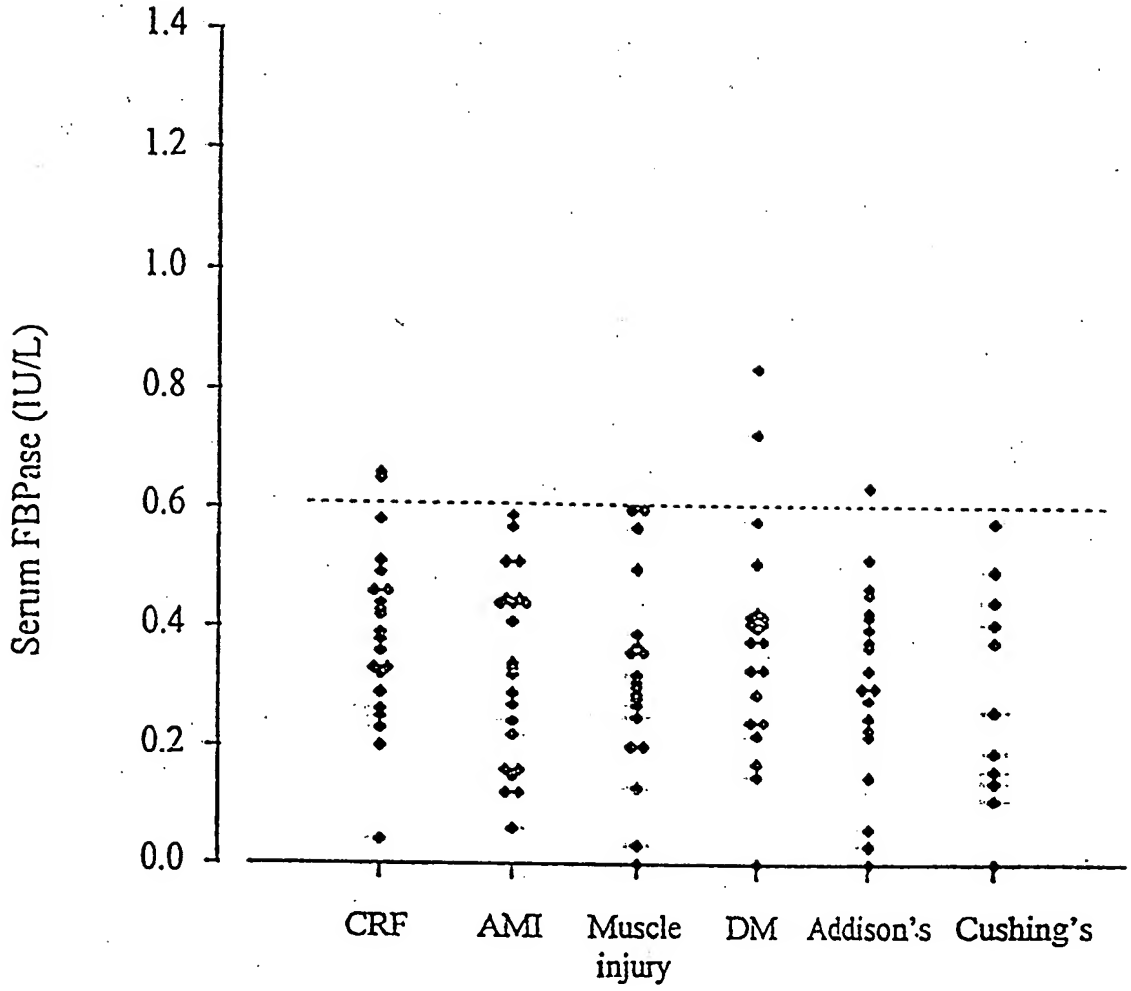
Serum FBPase (IU/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CRF
AMI
Muscle injury
DM
Addison's
Cushing's

Fig. 5

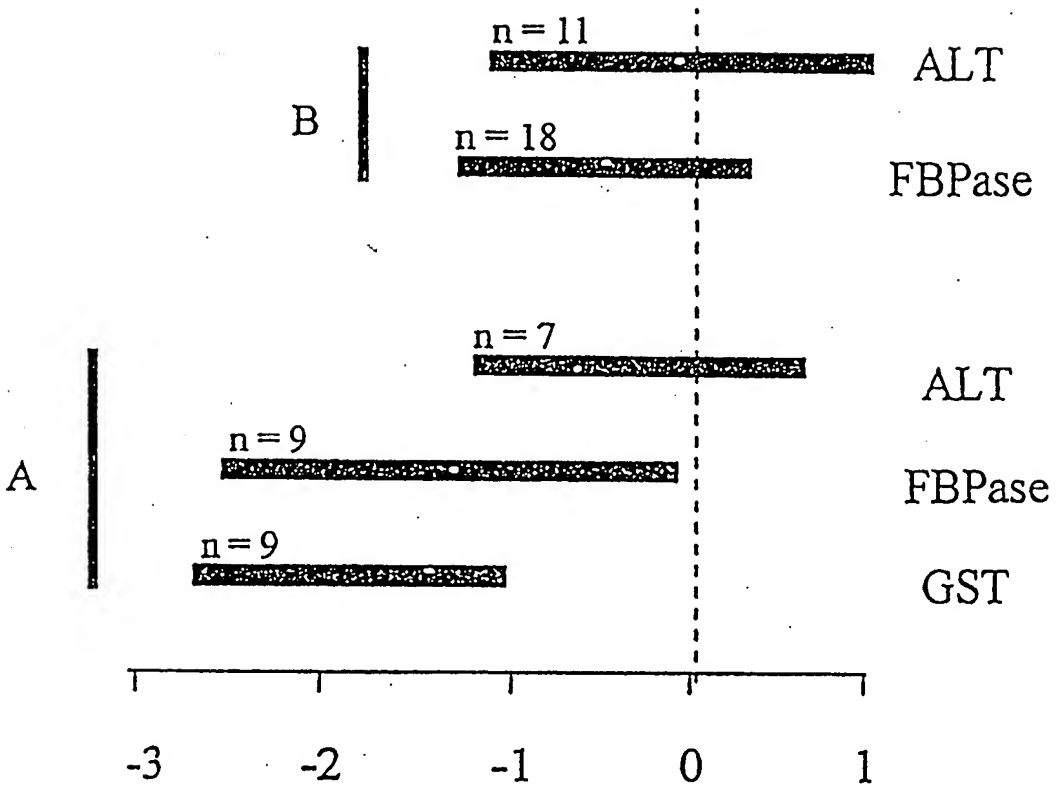
n = 11
ALT
B
n = 18
FBPase
n = 7
ALT
A
n = 9
FBPase
n = 9
GST
-3
-2
-1
0
1
Day relative to start of treatment

Fig. 6

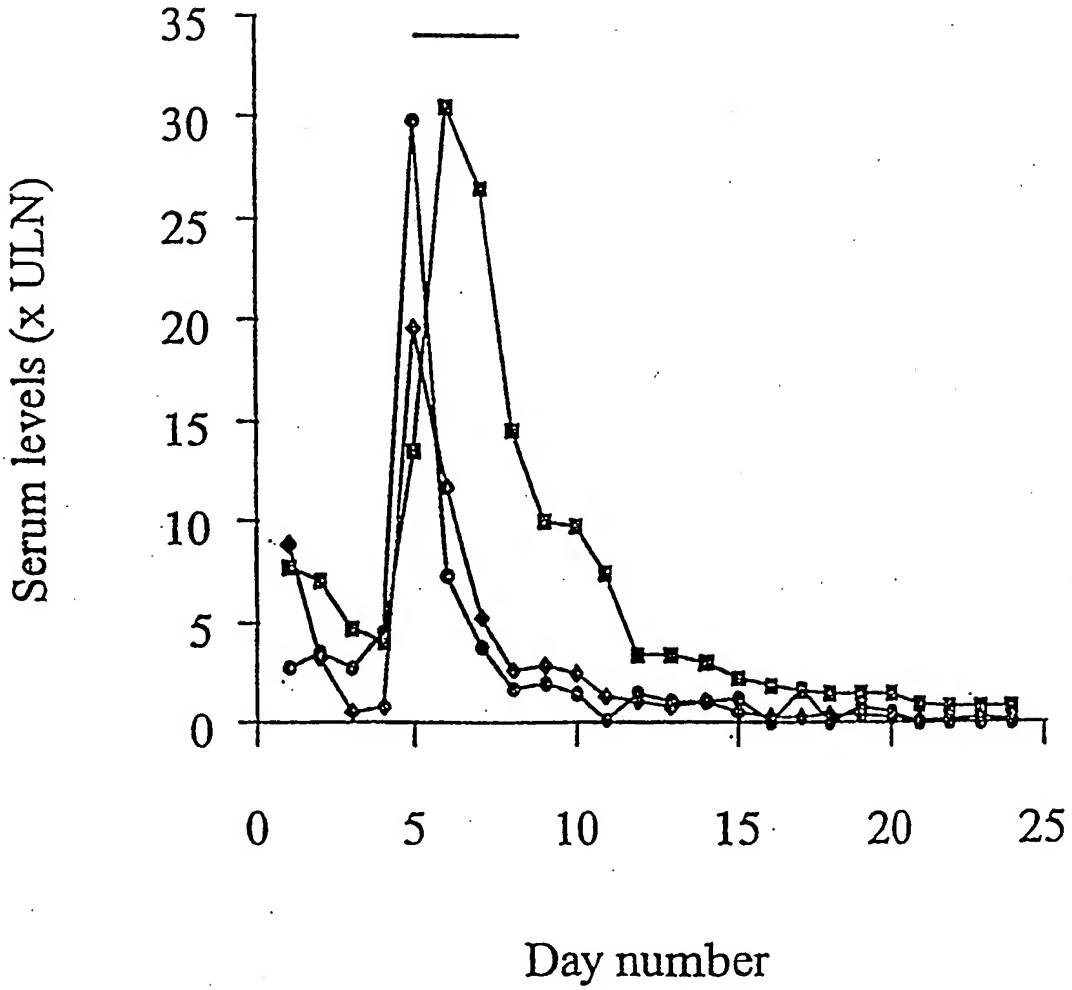

Fig. 7

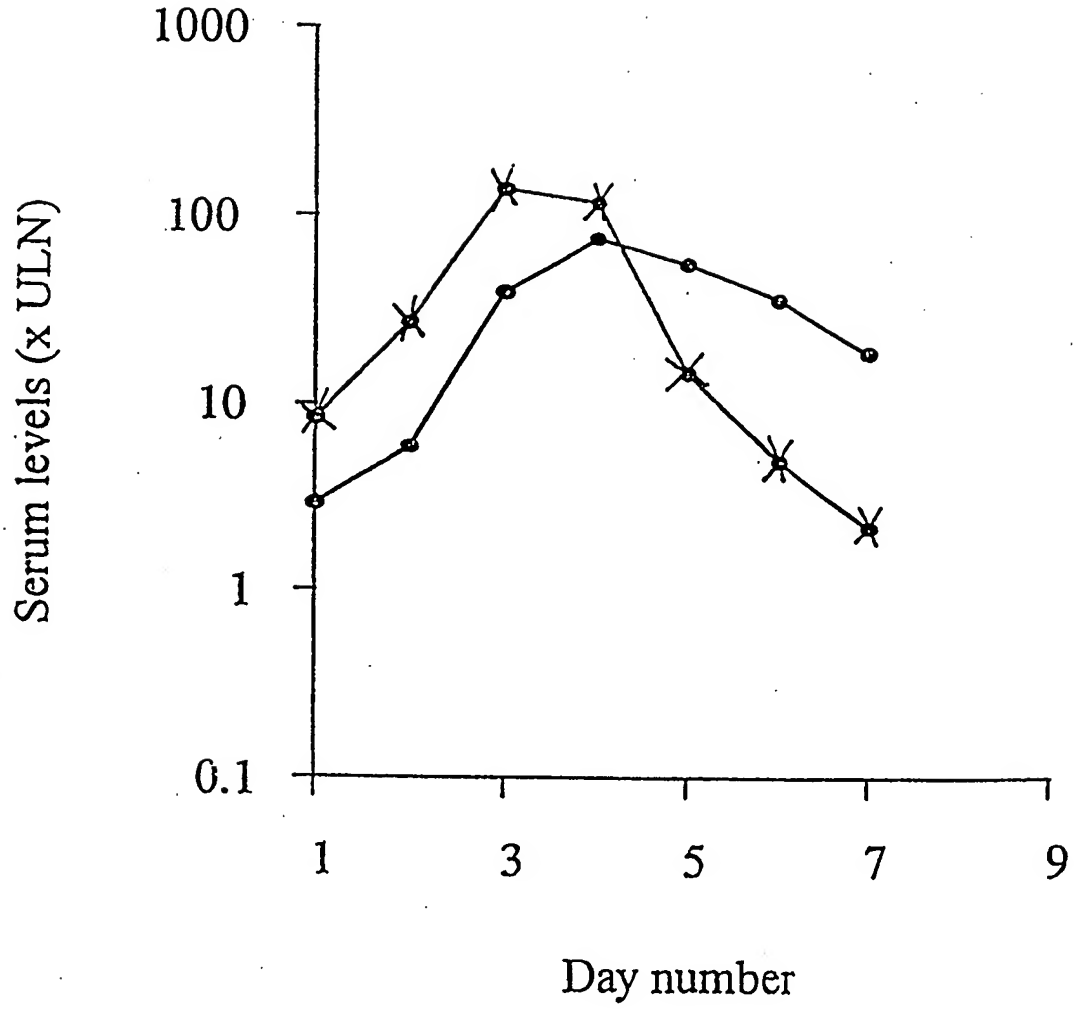

Fig. 8

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/EP 99/06216

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/42 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	BIOLOGICAL ABSTRACTS, Philadelphia PA USA; abstract no. PREV199900454185, abstract XP002125794 & A. MOROVAT ET AL.: "Serum fructose-1,6-bisphosphatase measurements for the early detection of acute hepatocellular damage " CLINICAL CHEMISTRY AND LABORATORY MEDICINE, vol. 37, no. supplement, 6 November 1999 (1999-11-06), page S472 Florence It --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
15 December 1999	28/12/1999

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 99/06216

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 112, no. 5, 29 January 1990 (1990-01-29) Columbus, Ohio, US; abstract no. 31877, XP002125795 cited in the application last sentence of abstract & P. MORATA ET AL.: "Fructose bisphosphatase activity in the serum of rats treated with carbon tetrachloride. " BIOCHEMISTRY INTERNATIONAL, vol. 19, no. 4, 1989, pages 863-869, New York NY USA ---	1-24
Y	CHEMICAL ABSTRACTS, vol. 100, no. 19, 7 May 1984 (1984-05-07) Columbus, Ohio, US; abstract no. 154811, XP002125796 cited in the application abstract & H. VON RECHENBERG ET AL.: " Fructose 1,6-bisphosphatase in the diagnosis of chronic hepatitis. II. Classification of chronic hepatitis based on fructose 1,6-bisphosphatase and other laboratory data" CLINICA CHIMICA ACTA , vol. 137, no. 3, 1984, pages 263-272, Amsterdam NL -----	1-24